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The cytotoxin of *Pseudomonas aeruginosa*: Cytotoxicity requires proteolytic activation

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Abstract. The primary structure of a cytotoxin from *Pseudomonas aeruginosa* was determined by sequencing of the structural gene. The cytotoxin (31,700 Mr) lacks an N-terminal signal sequence for bacterial secretion but contains a pentapeptide consensus sequence commonly found in prokaryotic proteins which function in a TonB-dependent manner. The cytotoxin gene has a [G + C]-content of 53.8% which is considerably lower than generally observed for genes from *Pseudomonas aeruginosa*. The cytotoxin gene was exclusively detected in strain 158 but not in three other clinical isolates, as determined by Southern and Northern hybridization. The latter technique revealed that the toxin is translated from monocistronic mRNA. The promoter of the cytotoxin is inactive in *Escherichia coli*. Upon site-directed modification of the 5′-noncoding region by the polymerase chain reaction the gene was expressed under control of the trc-promoter. The gene product obtained in *Escherichia coli* was nontoxic. Toxicity was induced by subsequent treatment with trypsin. [^{35}S]methionine-labeled cytotoxin with high specific radioactivity was obtained by in vitro transcription/translation. Like [^{125}I] labeled material from *Pseudomonas aeruginosa* this polypeptide bound to membrane preparations from Ehrlich ascites cells, as evidenced by sedimentation through a sucrose gradient at neutral pH.

Key words: *Pseudomonas aeruginosa* – Cytotoxin structure – Proteolytic processing – Ehrlich ascites cells

Pseudomonas aeruginosa is an opportunistic pathogen causing life-threatening disease in patients with weakened defense system by producing several toxic factors (Neu 1985). The cytotoxin has been characterized in autolysates of a *Pseudomonas aeruginosa* strain isolated from bovine mastitis milk. The protein accumulates in the periplasm of the bacterium (Kluftinger et al. 1989) and becomes liberated by autolysis rather than by secretion (Scharmann 1976). Isolated from bacterial autolysates, the cytotoxin has been characterized as a protein of 25,000 to 29,000 Mr which acts primarily on the plasma membranes of mammalian cells (Baltch et al. 1987; Kluftinger et al. 1989; Lutz 1979) by binding to a high affinity binding sites (Lutz 1986). As a consequence, pores of about 2 nm diameter (Lutz et al. 1987) are formed resulting in a breakdown of the cellular gradient for low molecular substances. The role of the cytotoxin in the manifestation of the *Pseudomonas aeruginosa* infection, however, has not been thoroughly investigated.

In this paper, we present the sequence of the cytotoxin. We show that a posttranslational activation step involving proteolytic removal of a 3,000 Mr peptide from the carboxy-terminal end takes place during or after autolysis.

Materials and methods

Materials. Enzymes were purchased from Boehringer (Mannheim, FRG). γ[^{32}P]ATP, α[^{35}S]dCTP, and L-[^{35}S]methionine were from Amersham (Braunschweig, FRG). Rabbit reticulocyte lysate was obtained from Amersham or Promega (Heidelberg, FRG), nucleotides and ribonuclease inhibitor were from Pharmacia (Freiburg, FRG), and anti-rabbit IgG from Dako (Copenhagen, Denmark). Diethylpyrocarbonate was from Sigma (München, FRG).

Bacterial strains and plasmids. Pseudomonas aeruginosa strain 158 (0:6; H:a_0, a_2, a_3; pyocin:38_c) was a clinical isolate from bovine mastitis milk. *Pseudomonas aeruginosa* strains 032 and 037 were isolated as swab samples from horse vagina or dog ear. The 054 strain was isolated from feces of a septicemic cow. All strains were propagated at 30°C on TSA/TSB (Difco, Detroit). *Escherichia coli* strains HB101 and JM101 were grown at 37°C in 2YT or M9-minimal medium. Plasmids pUC18/19 (Messing and Vieira 1982), M13mp18/19 (Norrander et al. 1983) and pRN653A,B,C (H. Niemann, A. Smid, M. Rosing, and E. Amann, unpublished) were used for establishing DNA-libraries, for DNA-sequencing, and for combined in vitro transcription/translation, respectively. For tightly regulated expression of the cytotoxin gene in *Escherichia coli* the IPTG-inducible vector pTrc99a (Amann et al. 1988) was used.

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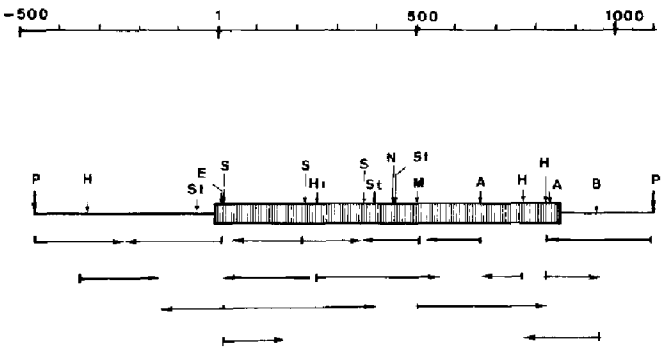

Fig. 1. Restriction map and sequencing strategy for the *Pseudomonas aeruginosa* cytotoxin gene. The hatched box represents the coding region. Restriction sites used for subcloning are indicated: *A*, AvaI; *B*, BalI; *E*, EcoRV; *H*, HpaII; *Hi*, HincII; *M*, MstI; *N*, NarI; *P*, PstI, *S*, Sau3AI; *St*, StyI. The horizontal arrows indicate individual M13-subclones and the direction of sequencing

Determination of the N-terminal amino acid sequence. Pseudomonas aeruginosa cytotoxin was purified from bacterial autolysate as described (Lutz 1979). The N-terminal amino acid sequence of the purified cytotoxin was determined by Edman degradation (Edman and Henschen 1975).

Hybridization conditions with synthetic oligonucleotides. Chromosomal DNA was isolated from logarithmically growing cultures as described (Meade et al. 1982). The mixture of heptadecamere oligonucleotides [ATGAA(C/T)GA(G/A)AT(C/T/A)-GA(C/T)AC] was synthesized with an Applied Biosystems model 380 A DNA synthesizer, 5′-labeled using γ[^{32}P]ATP and T4-polynucleotide kinase and used for hybridization according to Wallace et al. (1981).

Cloning procedures and DNA modifications. DNA modifications were performed according to standard protocols (Maniatis et al. 1982). The coding sequence for the N-terminus was identified with the 5′-labeled oligonucleotides on a 4 kb KpnI- and a 1.5 kb PstI-fragment. In addition, a signal was obtained with a 500 bp Sau3AI/PstI-fragment. The Sau3A/PstI-fragment was isolated from the gel and cloned under L3-B1 biosafety containment facilities into BamHI/PstI-digested pUC19. The insert was isolated by digestion with PstI and SmaI, nick-translated and used to screen PstI/HincII- and PstI/EcoRV-libraries. 12 overlapping M13 clones, together spanning the entire toxin gene (Fig 1), were sequenced on both strands to establish the complete structure employing the chain termination method (Sanger et al. 1977). Computer assisted analyses were performed with the PC-Gene program purchased from Genofit (Geneva, Switzerland).

Polymerase chain reaction (PCR). PCRs were performed with a Fischer robot (Fischerwerke, Tumlingen, FRG). For this purpose 10 ng of PstI-digested chromosomal DNA from *Pseudomonas aeruginosa* were amplified in 45 cycles with the two oligonucleotides (1 µM final concentration each), as shown in Fig. 2. The reaction mixture (100 µl) contained 2.5 U Taq-Polymerase (Boehringer, Mannheim) in 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01 gelatine and the four deoxynucleotides (each at 200 µM final concentration). Heat denaturation was for 1 min at 94° C; annealing of the oligonucleotides was performed at 45° C for 2 min, the polymerization reaction was at 72° C for 3 min. Through this procedure a singular FokI-site was introduced into the 5′-noncoding region. Subsequent cleavage with FokI generated 5′-CATG protruding ends that allowed cloning of the amplified gene into the NcoI-site of pTrc99a (Amann et al. 1988) to yield pSN3 as detailed in Fig. 2.

Expression of the cytotoxin in Escherichia coli. 10 ml precultures of *Escherichia coli* strain HB101 harboring pSN3 were prepared in LB-medium and used to inoculate 90 ml of TSB and 100 mg ampicillin per l. Cells were grown up to an optical density of OD660 nm of 1.0 and synthesis of the cytotoxin was induced by the addition of 1 ml of 0.5 mM IPTG. The incubation was continued for another 2 h at 37° C when cells were harvested by centrifugation at 16.000 × *g*, washed once with phosphate buffered saline (PBS), pH 7.4, and resuspended in 1 ml of PBS. The cells were incubated for 12 h at 37° C (autolysis step). Insoluble material was removed by centrifugation (30 min at 13,000 × *g*) and the supernatant was stored at −20° C. Toxicity assays were performed according to Gladstone and van Heyningen (1957). Proteolytic activation of the cytotoxin was achieved by the addition of 2.7% (w/w protein) TPCK-trypsin (40 U/mg, Boehringer) in lysates containing 3 mM (final concentration) of $CaCl_2$. After 2 h at 37° C reactions were stopped by the addition of 5-fold molar excess of trypsin inhibitor from soybean in 10 mM EDTA.

In vitro transcription/translations. The coding region of the cytotoxin gene was cloned from the EcoRV site (Fig. 3) on the 3′-PstI site into SmaI-PstI digested pRN653C to yield pOE65EP33. The sequence of the 5′-recombination site was verified by direct sequencing using the SP6 sequencing primer. Plasmid DNA was purified by two consecutive centrifugations on CsCl-density gradients. Transcriptions with SP6-polymerase and translations in rabbit reticulacyte lysate were performed as described previously (Mayer et al. 1988).

Interaction with plasma membrane preparations from Ehrlich ascites cells. Cytotoxin labeled during in vitro translation or by iodination was incubated for 2 h at 4° C and subsequently for 30 min at 30° C with plasma membrane preparations from Ehrlich mouse ascites cells (Kilberg and Christensen 1979). To assess membrane association, the incubation mixture was then placed on a sucrose cushion prepared in buffer that was either at pH 7.3 or at pH 11.0, as described in detail previously (Mayer et al. 1988). The pellet and the supernatant fraction were analyzed by SDS-PAGE.

Gel electrophoresis and immunoprecipitation. SDS-PAGE and processing with DMSO-PPO for autoradiography were performed as described (Niemann and Klenk 1980). Rabbit antibodies against cytotoxin were purified by binding the antibodies to nitocellulose carrying the purified toxin according to Burke et al. (1982). Protein A bearing *Staphylococcus aureus*, strain cowan I, was used to bind immune complexes.

Results

Determination of the primary structure of the cytotoxin of Pseudomonas aeruginosa

To establish the primary sequence of the cytotoxin from *Pseudomonas aeruginosa*, we have cloned and sequenced 12 overlapping hybridization-positive chromosomal DNA-fragments, as identified with an oligonucleotide probe reflecting all the possible codons for the N-terminal amino acid sequence (Fig. 1). Figure 3 shows a continous stretch of 1237 nucleotides containing a single open reading frame of 858 bp encoding a polypeptide of 286 amino acids with a molecular weight of 31,700. The sequence of the N-terminus was identical to the sequence determined by Edman degradation and the amino acid composition was in agreement with previous constituent analyses (Lutz 1979).

The hydropathy plot of the cytotoxin according to Kyte and Doolittle (1982) did not indicate hydrophobic

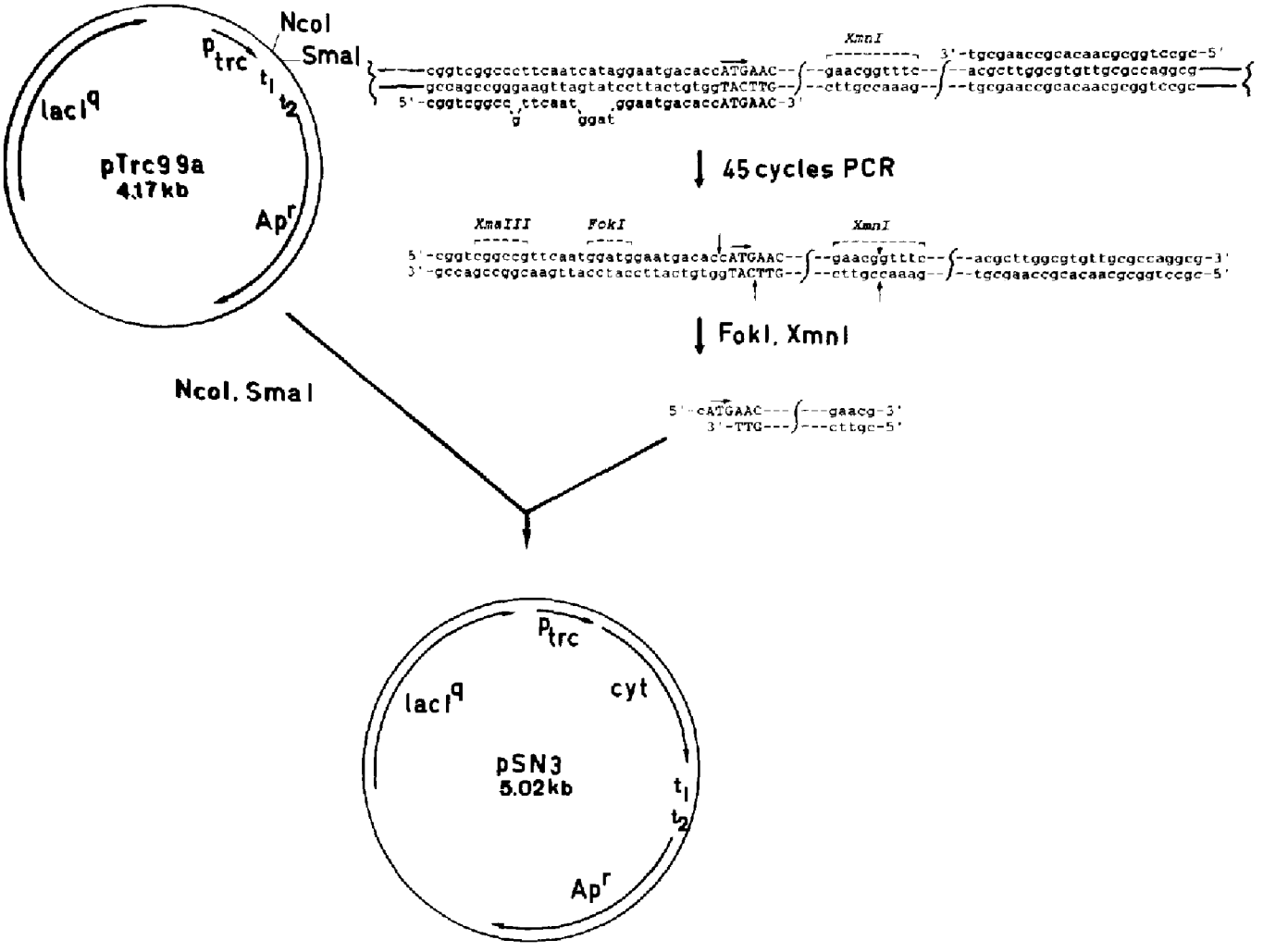

Fig. 2. Modification of the 5'-noncoding region of the cytotoxin gene using the polymerase chain reaction. The two oligonucleotides binding in the 5'- and 3'-noncoding regions (small letters) were annealed to PstI-digested chromosomal DNA from *Pseudomonas aeruginosa* as outlined in Materials and methods. The horizontal arrow indicates the translation start codon. Note that a mutation of the AAC-codon would lead to a mutation of Asn^2 and thus to a posttranslational removal of the N-terminal Met residue. Taq-Polymerase from Boehringer was used to introduce a singular FokI-site in 45 cycles. The products were digested with XmnI and FokI, purified by agarose gel electrophoresis and cloned into the SmaI/NcoI-digested pTrc99a (Amann et al. 1988)

domains that could be involved in catalyzing membrane integration. In addition, no signal sequence for secretion and no α-helical transmembrane domains were detected using the programs of Roa and Argos (1986) or Klein et al. (1985), respectively.

Organization and distribution of the cytotoxin gene

A Shine-Dalgarno consensus sequence (AGGA) was found 12 nucleotides upstream from the translation initiator ATG-codon. The [G + C]-content of the coding region (53.8%) is significantly lower than that reported for chromosomally integrated genes of *Pseudomonas aeruginosa* (West and Iglewski 1988), indicating that the gene could originally stem from a different organism. This hypothesis is further supported by our finding that the cytotoxin gene is absent in three other clinical isolates of *Pseudomonas aeruginosa* as evidenced by Southern analyses (Fig. 4A, B). Even after 45 PCR-cycles (using oligonucleotides binding immediately upstream and downstream from the coding region and 20 ng of chromosomal DNA) these other strains failed to produce a signal in Southern blotting. In addition, Northern blot analyses of RNA from the individual strains also indicated the absence of cytotoxin-specific transcripts (data not shown).

The open reading frame was followed by two inverted repeat structures indicated by divergent arrows in Fig. 3. The free energy values (Tinoco et al. 1973) of these stem-loop structures, −92.05 KJ/mol and −79.5 KJ/mol, suggest that they could function as transcription-termination signals. Northern blot analyses of RNA from strain 158 revealed that the cytotoxin-specific mRNA had a size of about 1100 nucleotides (data not shown). Taken together, these data support the conclusion that the cytotoxin gene is transcribed into monocistronic mRNA.

```
-120 CGGGTTTATTTTTTTTTCGCCAATACTAAGGCGTGGTTGTAGCCAAACAGTTCAGGAGCA
 -60 ATAACATAATAACTCCCAAGGTCAGATGGCGGTCGGCCCTTCAATCATAGGAATGACACC
                                              SD
   1 ATGAACGATATCGACACGATCACCAATGCGTGGGGACGTTGGAAAACGGCTCAATACGGA
       M  N  D  I  D  T  I  T  N  A  W  G  R  W  K  T  A  Q  Y  G
  61 ACCACCTGCTGGTTCACTGAAAGTACCCAGTACGGGCGGAACAAGGACACTCGCAGCTAT
       T  T  C  W  F  T  E  S  T  Q  Y  G  R  N  K  D  T  R  S  Y
 121 ATGCAACATCAGACCAACGTTTCCGCGCCGAAGGACTTGGTTTACTCCAACTTTGTGCAG
       M  Q  H  Q  T  N  V  S  A  P  K  D  L  V  Y  S  N  F  V  Q
 181 CAGGACGGCGGTAGCACCCTGTTGGGGCAGTACGACATGATCAACGAAGGCAGCCAAGTG
       Q  D  G  G  S  T  L  L  G  Q  Y  D  M  I  N  E  G  S  Q  V
 241 ATTGAACTTGCCGTCAACTTGCAACAAGGGTTAGTGGACACCTTCACCTGGAGCGTCACT
       I  E  L  A  V  N  L  Q  Q  G  L  V  D  T  F  T  W  S  V  T
 301 GAGCAGTTGAAGGTCGGTGTGGAAGTCAAGGTGAAGGCGAACATTCCCCTAGTGGGCGGC
       E  Q  L  K  V  G  V  E  V  K  V  K  A  N  I  P  L  V  G  G
 361 GCTGAGATCACCAGTACGGTGGAATTGTCACTGTCCTCTACCCAAGGGGCGAGTACCAGC
       A  E  I  T  S  T  V  E  L  S  L  S  S  T  Q  G  A  S  T  S
 421 AAGTCTTCCAACTATGGCGCCTCTACCAAGGTGCTTATTTCCCCACATAGCCACGGCTGG
       K  S  S  N  Y  G  A  S  T  K  V  L  I  S  P  H  S  H  G  W
 481 GGAGAGGTTGCCTTGAGCTTTACTGAGCTGCGCAGTCAGTGGGTCGGTAATGTCGGGCTT
       G  E  V  A  L  S  F  T  E  L  R  T  Q  W  V  G  N  V  G  L
 541 CAAGGATATGTGGCAATTTGGTTCAACAACAAAGTCGCATTGAACAACGATGGCGATTAC
       Q  G  Y  V  A  I  W  F  N  N  K  V  A  L  N  N  D  G  D  Y
 601 CACTACCTGTGGTTCATTCCCGTGGAGCAGGTATTTTGGGAGTGCGTCCAGCACAACATA
       H  Y  L  W  F  I  P  V  E  Q  V  F  W  E  C  V  Q  H  N  I
 661 GTCAATACCTCGGGCTATGTCGTACAAGGCAATGGAGTGTTGGCGCAAGCCACAGGCACC
       V  N  T  S  G  Y  V  V  Q  G  N  G  V  L  A  Q  A  T  G  T
 721 TTCCATAGCAGCGTGGGCTTGAACTTGAAGACCATCGCGCACGAGCGGCGCTATCCGGAA
       F  H  S  S  V  G  L  N  L  K  T  I  A  H  E  R  P  Y  P  E
 781 ACCTCGGAGGCGGTCAGAACATTCTATAATTATGCCAGTCTGGTTCCGGACCTAGAAACC
       T  S  E  A  V  R  T  F  Y  N  Y  A  S  L  V  P  D  L  E  T
 841 CGAGTCCGCTCGGCAGAGTAGAAAGCCGTAGGAAGTCTGGAACGGTTTCTGTCCGTTAAA
       R  V  R  S  A  E  ***
 901 CGTAGAACGCTTGGCGTGTTGCGCCAGGCGTTACAGGTTGCTGACAAAGCCCTGGCCACA
       <----- -----    ----- ---->          <----- -------
                   -92.05                          -79.5
 961 AGGCCAGGCTTTGTATTTAGTGATTCCTCATCTTCTCCCTAGTACCCCTTGCTCGGACAG
       ------------>
1021 CTTTCTCCTTTACGGGGTGATATTGGAGACGCTGATTAGAAGCCTGGAGCGTCGAACTGC
1081 GCGAACCTCACGACCTCCTCCCCGATCATCTCATTAA
```

Fig. 3. Sequence of the cytotoxin gene from *Pseudomonas aeruginosa*. The putative Shine Dalgarno sequence is underlined Divergent arrows in the 3′-noncoding region represent stem-loop structures which could serve as signals for termination of transcription. Numbers indicates free-energy values in KJ/mol calculated according to Tinoco et al. (1973)

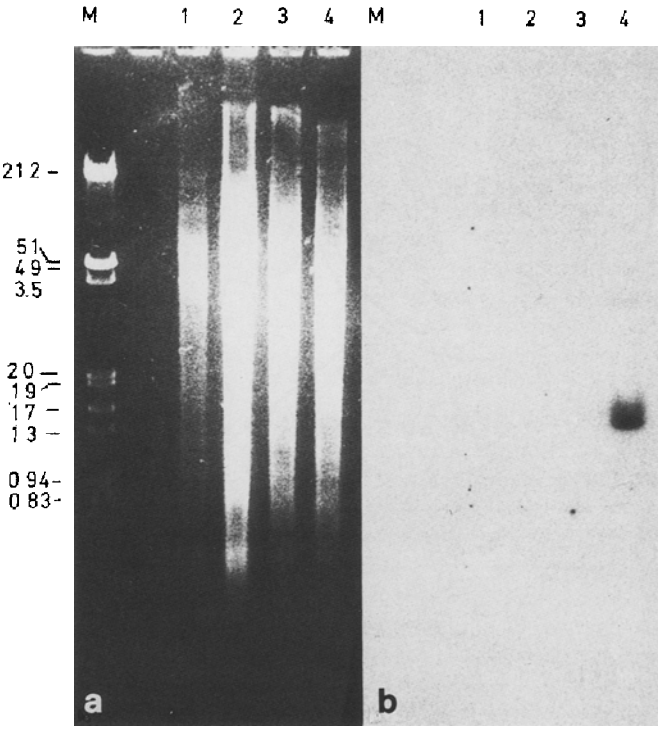

Fig. 4 a,b. Distribution of the cytotoxin gene in individual clinical isolates of *Pseudomonas aeruginosa*. **a** Southern analyses of chromosomal DNA isolated from various *Pseudomonas aeruginosa* strains. Chromosomal DNA was isolated from strains 032 (*lane 1*), 037 (*lane 2*), 054 (*lane 3*), and 158 (*lane 4*), digested with PstI, and analyzed on a 0.8% agarose gel together with EcoRI/HindIII digested lambda DNA (*lane M*). After transfer onto a nitrocellulose sheet cytotoxin specific fragments were detected with a nick-translated EcoRV-PstI fragment from pSN3

Expression of the cytotoxin gene in vitro and in Escherichia coli

To test the properties of the cloned gene product, we expressed the gene in vitro or in *Escherichia coli* and compared the properties of the products with those of unlabeled or [^{125}I] labeled cytotoxin, as isolated from *Pseudomonas aeruginosa*. For combined in vitro transcription translations, we cloned the coding region from the EcoRV site (cleavage after position 9 of the coding region) to the PstI-site into a modified pSP65-vector system providing an ATG codon for initiation of translation. The resulting construct, pOE65EP33, yielded mRNA encoding a polypeptide of 30,000 Mr which lacked the three N-terminal amino acids of the toxin and contained 8 forcigns residues encoded by the polylinker region. Translation of the RNA in rabbit reticulocyte lysate produced a major polypeptide of 30,000 Mr (Fig. 5A, lane 1). This molecular species had an electrophoretic mobility that was indistinguishable from material isolated from intact *Pseudomonas aeruginosa* cells (compare lanes 1 and 2).

As demonstrated by Western blotting, the 30,000 intracellular form of the cytotoxin (lane 3) migrated clearly slower in SDS-PAGE than the 28,000 material that was isolated from autolysates (lane 4). Puls-chase experiments of [^{35}S]methionine labeled sister cultures of *Pseudomonas aeruginosa* did not reveal a conversion of the 30,000 species into the 28,000 species (data not shown) indicating that the putative processing step had to occur during autolysis of the bacteria. Expression of the cytotoxin gene in *Escherichia coli* was inducible with IPTG (compare lanes 1 and 2 in Fig. 5B), again yielding material that migrated like the non-processed form of the cytotoxin in SDS-PAGE (compare with lane 4). This material was clearly nontoxic in the granulocyte lysis assay (Fig. 6A). As shown in lanes 2 and 3 of Fig. 5B, treatment of *Escherichia coli* lysates with trypsin converted the 30,000 species into two smaller species of 28,000 and 26,000 (lane 3). Concomitantly a rapid increase in toxicity was observed (Fig. 6B), indicating that the removal of the

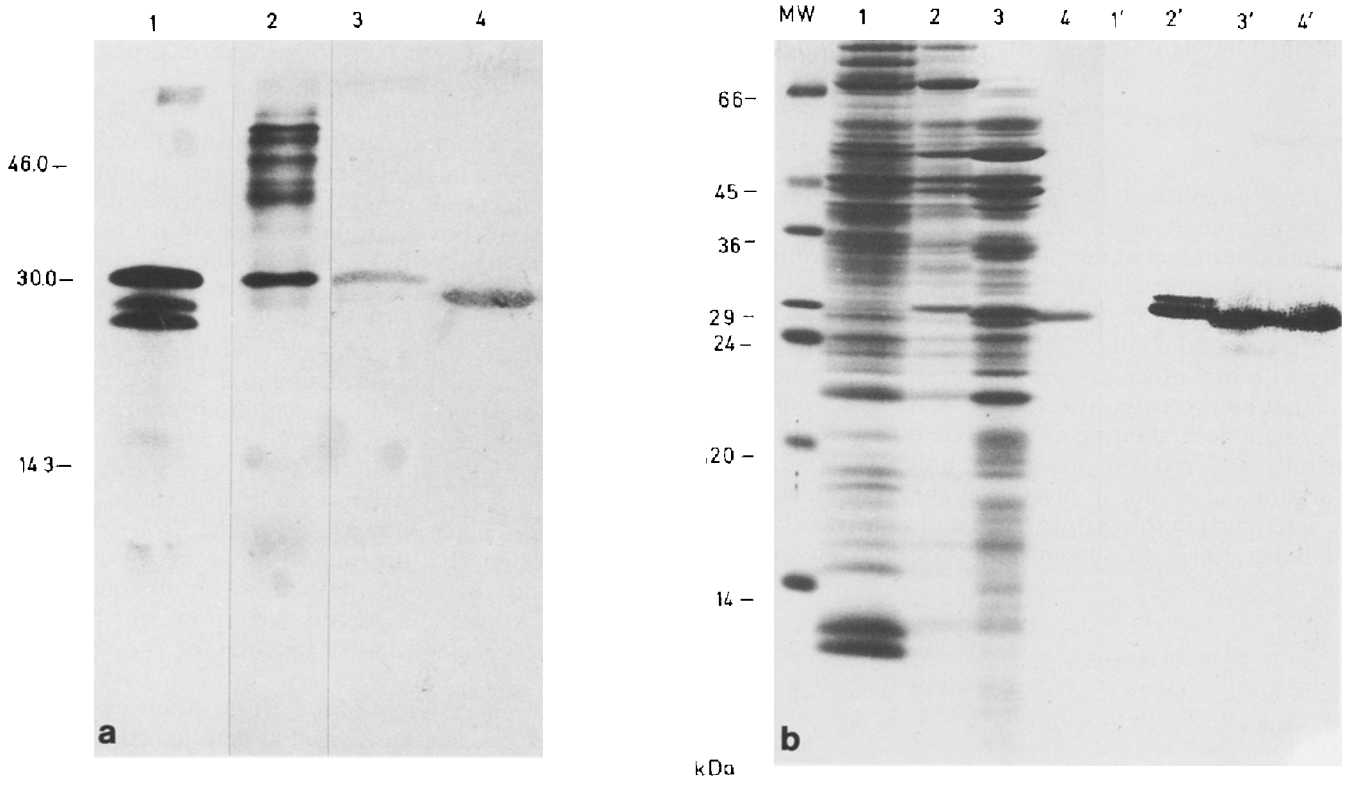

Fig. 5a, b. Comparison of in vitro synthesized cytotoxin with intracellular material from *Pseudomonas aeruginosa* or from recombinant *Escherichia coli*. **a** Cytotoxin-specific mRNA was transcribed in vitro from purified pOE65EP33-DNA using SP6 RNA-polymerase. Translations were performed in rabbit reticulocyte lysate in the presence of L-[^{35}S]methionine. The products were immunoprecipitated with a cytotoxin-specific polyclonal rabbit serum (*lane 1*). For comparison metabolically labeled cytotoxin was liberated from intact bacteria by repeated freezing and thawing and ultrasonification. The lysate was subjected to immunoprecipitation (*lane 2*). Lanes 3 and 4 show a comparison of the cellular form of the cytotoxin (*lane 3*) with cytotoxin from autolysates (*lane 4*) **b** Expression in *Escherichia coli* and proteolytic activation of the cytotoxin. HB101 cells harboring the plasmids pTre99a (*lane 1*) or pSN3 (*lanes 2, 3*) were grown in TSB, treated with IPTG, and lysed as described in Materials and methods. Samples were analysed in duplicate together with cytotoxin from pseudomonal autolysates (*lane 4*) by SDS-PAGE. The gel was divided and either stained with Coomassie brilliant blue (*left panel*) or subjected to Western blotting (*right panel*) using an affinity purified polyclonal rabbit serum against cytotoxin

short peptide sequence led to the activation of the cytotoxin.

Binding properties of the cytotoxin to plasma membrane preparations from Ehrlich ascites cells

To see whether this proteolytic processing step altered the binding properties of the cytotoxin to cellular receptors, we performed binding studies of the in vitro synthesized cytotoxin derivative and compared it with iodinated cytotoxin as derived from *Pseudomonas aeruginosa* autolysates. Binding of the cytotoxin to membrane preparations was assessed by co-sedimentation of the radiolabeled cytotoxin with the membranes through a sucrose cushion of neutral pH. The results are summarized in Fig. 7. No difference was detected in the binding properties of the in vitro synthesized full-size cytotoxin and the processed cytotoxin. In both instances binding was reversible by the addition of a 100-fold excess of unlabeled cytotoxin (data not shown). However, binding apparently involved only attachment to peripheral binding sites, since a significant amount of labeled material

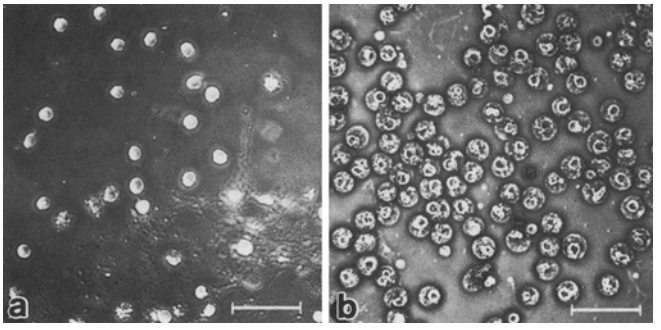

Fig. 6. Toxicity assays of *Escherichia coli* derived cytotoxin on granulocytes before (**a**) and after (**b**) treatment with trypsin

eluted from the membranes, when the pellet fraction was resuspended and re-sedimented through the sucrose cushion (compare lanes 9 and 10 of Fig. 7). Furthermore, no co-sedimentation of the labeled material was observed when the sucrose was made up in buffer of pH 11.0, again indicating that the bound material was not converted into an intrinsic membrane protein. It is clear, however, that

such observations have to be confirmed by experiments involving binding to intact cells.

Discussion

We have established the sequence of a pore-forming cytotoxin from *Pseudomonas aeruginosa* by determining the amino-terminal amino acid sequence of the purified protein and sequencing of the structural gene, as identified by a pool of synthetic oligonucleotides. The cytotoxin sequence did not reveal a significant sequence similarity with any other known protein. It is important to note that no proteolytic processing of the N-terminus of the protein occurs during or after bacterial autolysis. As generally observed with procaryotic polypeptide carrying an asparagine residue in position 2, the methionine residue is retained in the mature toxin molecule (Ben-Bassat and Bauer 1987). In agreement with a previous report (Scharmann 1976) indicating that the cytotoxin was released from the bacteria only after several days of growth, the molecule lacks a secretory signal.

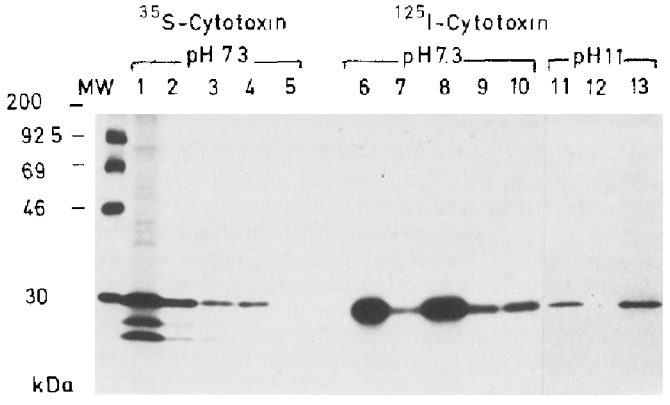

Fig. 7. Binding of cytotoxin to plasma membrane vesicles from Ehrlich ascites cells. In vitro translated cytotoxin (*lane 1*) or [^{125}I]-labeled cytotoxin (*lanes 6* and *11*) were incubated with plasma membrane preparations and sedimented together with the membranes through a sucrose cushion at the pH indicated (Mayer et al. 1988). The pellet fractions (*lanes 2, 4, 7, 9,* and *12*) and TCA-precipitable material from the supernatant fractions (*lanes 3, 5, 8, 10,* and *13*) were analysed by SDS-PAGE. Samples in *lanes 4* and *5*, and *9* and *10* are derived from a second centrifugation step

Within the N-terminal domain, a remarkable homology to a pentapeptide consensus sequence (TonB-box), commonly found in outer membrane receptor proteins of the *Escherichia coli* iron transport system, was detected. As yet, the TonB-box was found exclusively in all proteins that function in a TonB-dependent manner (Braun et al. 1987). Interestingly, this group of proteins contains also some colicins known to kill closely related bacteria by pore formation. Uptake of such colicins by the target cell occurs in a receptor mediated and TonB-dependent process (Braun et al. 1987). Recent modifications of the TonB-box from the FhuA receptor by site-directed mutagenesis (Schöffler and Braun 1989) have shown that a replacement of the Val^{11} residue by aspartic acid only weakened the colicin M sensitivity of the *Escherichia coli* strain indicating that the interaction between the FhuA receptor and the TonB protein was not completely abolished. At present, we do not know whether the cytotoxin serves a colicin-like function for *Pseudomonas aeruginosa*.

The molecular weight of the cytotoxin purified from bacterial autolysates was 28,000 as determined by SDS-PAGE. This material migrated clearly faster than the 30,000 Mr species obtained by in vitro transcription/translation or by expression in *Escherichia coli*. Although the in vitro synthesized material bound specifically to membrane preparations from Ehrlich ascites cells, exhibiting properties indistinguishable from the mature [^{125}I] labeled cytotoxin, this non-processed form was nontoxic in the granulocyte lysis assay. Cytotoxicity clearly required proteolytical processing which in autolysates was mediated by endogenous proteases. Trypsin-treatment of *Escherichia coli* lysates also restored cytotoxicity. Such processing could involve only C-terminal sequences, since identical N-termini were determined by Edman degradation and by DNA-sequencing. The mechanism by which pore formation through the cytotoxin is induced is far from being understood at the molecular level. We show here that binding to peripheral acceptor sites does not require proteolytic processing and

Table 1. Sequence homology of the cytotoxin from *Pseudomonas aeruginosa* with various TonB-dependent proteins from *Escherichia coli*. The TonB-specific sequences are framed

Protein	Number of the first residue shown	TonB sequence	Reference
Cytotoxin	4	IDTITNAW	this study
Fhu A	6	EDTITVTA	Coulton et al. 1986
Fhu E	5	EETITVTA	Sauer et al. 1987
Btu B	25	PDTLVVTA	Heller and Kadner 1985
Fec A	22	GFTLSVDA	Pressler et al. 1988
Fep A	11	DDTIVVTA	Lundrigan and Kadner 1986
Cir	5	GETMVVSA	Griggs et al. 1987
Iut A	5	DETFVVSA	Krone et al. 1985
Colicin M	1	METLIVEA	Köck et al. 1987
Colicin B	16	GDTMVVWP	Schramm et al. 1987
Colicin 1 b	22	HEIMAVDI	Mankovich et al. 1984

also does not involve the N-terminal sequences, since the in vitro synthesized cytotoxin had similar binding properties like the mature molecule. With the cytotoxin gene at hand and the development of various deletion mutants thereof further studies on the pore formation process can now be undertaken.

Appendix. While this manuscript was in preparation, Hayashi et al. (1989) published their data on the nucleotide sequence and the expression of the cytotoxin gene. The authors also came to the conclusion that cytotoxicity was posttranslationally generated by proteolytic cleavage.

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